The development and characterization of neural-specific continuous cell lines in *Drosophila melanogaster*

Undergraduate Research Thesis

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Abstract

Neurodegenerative diseases affect millions of Americans and are caused by the deterioration of neurons in the central nervous system (CNS). The mutations that cause these neurodegenerative diseases have been modeled in *Drosophila melanogaster* (fruit fly) transgenic lines that produce flies with neurological defects mirroring the human pathology. Meanwhile, continuous cell lines have transformed biomedical research, making it possible to study biological systems on a scale requiring large numbers of similar cells. However, there is a lack of continuous cell lines of neuronal lineage in *Drosophila melanogaster* available in the scientific community that would allow for the modeling of neurodegenerative diseases and the detailed study of specific mechanisms within the nervous system. Beyond modeling a neurodegenerative disease phenotype in a fly, continuous cell lines of neuronal lineage would offer a large, homogenous population of cells to potentially study disease pathways and gene function, to test pharmacological targets, and to examine processes occurring before differentiation. The primary objective of this study was to utilize the expression of oncogenic Ras^{V12} and inhibition of the Notch signaling pathway to produce neuronal cell lines in *Drosophila melanogaster*. In the first approach, the overexpression of Ras^{V12} was limited to cells of neuronal lineage in order to promote their survival over other cell types in the culture. In the second approach, the Notch signaling necessary for segregation of progenitor cells into neuroblasts or epithelial cells was inhibited throughout the *Drosophila* embryo; without Notch signaling, these progenitor cells all become neuroblasts. For the second objective of the study, cell lines previously established in the lab from constitutive Ras^{V12} expression and that appeared to exhibit neuronal properties were characterized with neuronal lineage markers. This study tests the efficacy of utilizing Ras^{V12} or

the inhibition of the Notch signaling pathway for the production of continuous cell lines of neuronal lineage to model and develop therapies for neurodegenerative diseases.

Acknowledgements

I would like to thank the members of the Simcox Laboratory for their mentorship and guidance over the past three years (Shiva Raghuvanshi, Ashley Heinman, Geeta Palsule, Dr. Uyen Tram, and Dr. Sathiya Manivannan). In particular, I would like to thank Dr. Amanda Simcox for giving me the opportunity to be a part of her laboratory, for teaching me how to become a better student researcher, and for her support throughout my undergraduate career. I would also like to thank Dr. Mark Seeger and Dr. Cynthia Bronson for serving on my committee, and the College of Arts and Sciences for the 2015 and 2016 Undergraduate Research Scholarships to support my research.

Introduction

Immortal cell lines are an effective and commonly used tool to study biological processes. In comparison to primary cells, which are directly derived from tissue, or in vivo models, cell lines are more cost effective, easy to use, and provide a large, consistent population of cells for study (Kaur and Dufour 2012). Additionally, the use of cell lines does not require repeated use of animal or human tissue, thus circumventing ethical concerns (Kaur and Dufour 2012). Over the past few decades, the utility of cell lines has transformed scientific research, allowing for the production of vaccines, drug screens, antibody production, and assessment of gene function (Kaur and Dufour 2012). Cell lines of a specific lineage representing a disease phenotype have also been used to study disease mechanisms and to test potential pharmacological targets (Schlachetzki *et al.* 2013).

Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, are progressive disorders of the central and peripheral nervous systems (Schlachetzki *et al.* 2014; Lu and Vogel 2011). Many of these disease phenotypes have been modeled and studied in *Drosophila* due to the presence of several neurodegenerative disease-associated gene homologs in the model organism (Lu and Vogel 2011; Bilen and Bonini 2005). Though there are some neural-like cell lines derived from the *Drosophila* central nervous system, these existing lines do not have clear neuronal character (Ui-Tei and Miyata 2000). In comparison to studying the nervous system in the fly itself, neuronal lineage-specific cell lines could be utilized to analyze cells of neuronal lineage before differentiation, to efficiently study neurodegenerative disease-associated genes and pathways, and to potentially test pharmacological targets for these diseases. Though mammalian cell lines, such as those derived from human neuroblastoma, are used by scientists to study

neurodegenerative diseases, a cell line from *Drosophila* would offer a population of cells without the added redundancy and complexity of a mammalian genome (Xicoy *et al.* 2017).

The goal of this study was to develop neuronal continuous cell lines from *Drosophila* using two primary approaches. The first approach uses an established method from the Simcox Lab in which the oncogene Ras^{V12} is overexpressed, promoting the survival of cells (Simcox *et al.* 2008, Simcox 2013). To generate neuronal cells specifically, Ras^{V12} expression was limited to neuronal cells by using the Gal4/UAS system, which allows for targeted gene expression in a tissue-specific manner (Brand and Perrimon 1993). In limiting the Ras^{V12} expression to neuronal cells, these cells are provided a survival advantage over other cells present in the primary culture, allowing the neuronal cells to become the predominant cell type in the culture (Simcox 2013).

Crosses were set up between Gal4 driver flies, which express the Gal4 transcription factor protein in a specific pattern, with UAS responder flies, which contain an Upstream Activating Sequences (UAS) element upstream of the Ras^{V12} oncogene (Duffy 2002 and Simcox *et al.* 2008). To produce neuronal-specific and motor neuron-specific primary cultures, I used a pan-neuronal Gal4 driver and two motor neuron-specific Gal4 drivers respectively. The pan-neuronal Gal4 driver was *scratch-Gal4,* which is expressed in most or all neuronal precursor cells and is involved with the development of the *Drosophila* nervous system (Roark *et al.* 1995). The motor neuron-specific Gal4 drivers were *OK6-Gal4* and *Toll-6-Gal4;* whereas *OK6-Gal4* expression is primarily restricted to motor neurons, *Toll-6-Gal4* is also expressed in the peripheral nervous system and the body wall sensory nervous system (Sanyal 2009). These drivers were crossed with a number of UAS responder lines, all expressing oncogenic Ras^{V12}.

The second approach attempted to culture neuroblasts through inhibition of the Notch signaling pathway. Notch signaling plays a critical role in the development of the nervous system

in *Drosophila* and has been found to regulate binary fate determination of progenitor cells in the *Drosophila* embryo; specifically, Notch signaling has been found to influence the segregation of cells into neural and epidermal cell lineages (Louvi and Artavanis-Tsakonas 2006; Cau and Blader 2009). The complete deletion of *Notch* was found to prevent the segregation of cells into the neural and epidermal cell types, with all cells instead becoming neuroblasts at the expense of epidermal cells (Louvi and Artavanis-Tsakonas 2006). Notch null mutant *Drosophila melanogaster* embryos were also shown to exhibit hypertrophy of the central and peripheral nervous systems (Louvi and Artavanis-Tsakonas 2006). In this study, the Gal4/UAS system was used to produce embryos with Notch signaling inhibited in many cell types. To do this, *Act5C-Gal4* flies, with Gal4 in cells expressing the cytoplasmic actin gene Act5C, were crossed with *UAS-Notch*^{dsRNA} flies.

Lastly, previous studies in the Simcox Lab crossed *Act5C-Gal4/TM6* flies with *UAS-Ras*^{V12}*; P[attP.w*$^{+}$*.attP]JB89B/TM3* flies to produce two continuous cell lines with constitutive Ras^{V12} expression in many cell types: Ras-attP-L1 and Ras-attP-L2 (Manivannan *et al.* 2015). Despite the expression of Ras^{V12} in many cell types, these cell lines appeared to be neuronal in morphology, with axon- and dendrite-like projections (Rolls 2011). I characterized these cell lines using neuronal lineage markers and found the two cell lines to be of neuronal lineage.

Materials and Methods

Fly Stocks:

The following fly stocks were used: *scratch-Gal4* (Pfieffer *et al.* 2008, Bloomington Stock Center #47368); *OK6-Gal4* (Bloomington Stock Center #64199); *Toll-6-Gal4* (Bloomington Stock Center #8816); *Actin5C-Gal4/TM6b, Tb; UAS-Notch^{dsRNA}* (Bloomington Stock Center #7078); *UAS-Ras^{V12}; UAS-GFP; UAS-Ras^{V12}, RMCE; UAS-p35; UAS-Ras^{V12}, RMCE; and UAS-CD8-GFP.*

Production of Primary Cultures:

Cages of approximately 200 males and 200 virgin female flies from the Gal4 drivers and UAS responder lines were crossed in well-yeasted vials using an established method (Simcox 2013). The flies were kept overnight and then transferred to a cage containing sterile egg-collection plates from which embryos were collected every 7 to 12 hours at room temperature. Eggs were transferred from the plate to a 15 mL conical tube using TXN and dechorionated in 50% bleach under the hood. After subsequent washes in TXN, sterile water, and cell culture medium, the eggs were homogenized. The homogenate was centrifuged, the supernatant was removed, and then the pellet was plated as a primary culture in a 25 cm^3 flask. The flasks were maintained in an incubator at 22 °C and the medium was changed about every 7 to 10 days. Cells were passaged to a 70 cm^3 flask at 90 percent confluence.

Visualization of Gal4 Expression in Drosophila Embryos and Larvae:

The pan-neuronal Gal4 driver, *scratch-Gal4*, and the motor neuron Gal4 drivers, *OK6-Gal4* and *Toll-6-Gal4*, were crossed with *UAS-Ras^{V12}; UAS-GFP* flies to visualize the expression pattern

of the given Gal4 driver with green fluorescent protein (GFP) in progeny. Embryos were collected from the cross, dechorionated in 50% bleach, and then imaged using fluorescent microscopy. To visualize the expression pattern of the three Gal4 drivers in larvae, the drivers were crossed with *UAS-CD8-GFP* flies. Images were taken of progeny larvae at the first instar, second instar, and third instar larval developmental stages.

Immunostaining of RR1 and RR2 Cell Lines:

Cells were plated on chamber slides, fixed in 4% paraformaldehyde solution, incubated with the primary antibodies 22C10, Fasciclin-II, and horseradish peroxidase (HRP), and then imaged with fluorescent microscopy. 22C10 and Fasciclin-II were used at 1:100 dilution, and HRP was used at 1:500 dilution. Cells stained with 22C10 and Fasciclin-II were incubated with anti-mouse secondary antibody at 1:1000 dilution. Cells stained with HRP were incubated with anti-rabbit secondary antibody at 1:1000 dilution. The chamber slides were mounted in VectaShield® Mounting Medium with DAPI (Vector Laboratories).

Results

Selection of Gal4 lines for expression of Ras^{V12} in the nervous system

In order to direct Ras^{V12} expression broadly with the nervous system, or in specific cell types, a number of Gal4 lines with different expression patterns were considered based on their known expression patterns or by using expression of the reporter gene, UAS-GFP. The Gal4 lines that were initially surveyed included the Janelia Gal4 stocks described in Pfeiffer *et al*. 2011 and the GeneSwitch-Gal4 drivers described in Nicholson *et al.* 2008. The GeneSwitch-Gal4 system allows for the temporal control of Gal4 expression in the presence of the steroid RU486 (Nicholson *et al.* 2008). This system can be used in conjunction with a responder line expressing Ras^{V12} to selectively induce Ras^{V12} expression in cell culture (Simcox and colleagues, unpublished). Of the surveyed Gal4 lines, seven drivers with specific expression within the *Drosophila* nervous system were selected to be potential drivers to produce neural-specific continuous cell lines. In addition, a survey of the literature on motor neuron-specific Gal4 drivers led to the selection of *OK6-Gal4* and *Toll-6-Gal4* as drivers for the production of motor neuron-specific continuous cell lines. Descriptions of the Gal4 and GeneSwitch-Gal4 drivers are listed in Table 1.

Driver Line	Gal4 Expression Pattern
GSG3147-1 (Bloomington Stock Center #40315)	Primarily neuronal expression and strong expression in sensory neurons; weak expression in the ventral nerve cord; some expression in imaginal discs (Nicholson *et al*. 2008)
GSG5793 (Bloomington Stock Center #40333)	Primarily neuronal expression with strong expression in sensory neurons, weak expression in the ventral nerve cord, and moderate expression in the brain (Nicholson *et al*. 2008)
GSG686 (Bloomington Stock Center #40981)	Primarily neuronal expression with moderate expression in neuromuscular junction neurons, and some expression in the brain and ventral nerve cord (Nicholson *et al.* 2008)
Deadpan-Gal4 (Bloomington Stock Center #47456)	Pan-neuronal driver; strong expression in neuroepithelium cells and medulla neuroblasts, and weak expression in medulla neurons (Yasugi et al. 2014 for Bloomington Stock Center #47456)
Deadpan-Gal4 (Bloomington Stock Center #47859)	
Scratch-Gal4 (Bloomington Stock Center #38761)	Expresses Gal4 under the control of DNA sequences in or near *scrt*; the *scrt* gene is pan-neural and is expressed in most or all neuroblasts in *Drosophila* (Pfeiffer *et al*. 2011, Roark *et al.* 1995)
Scratch-Gal4 (Bloomington Stock Center #47368)	

Table 1. Selected Gal4 and GeneSwitch-Gal4 drivers exhibit specific expression throughout the *Drosophila* nervous system.

Previous research from the Simcox Lab attempted to create neural-specific continuous cell lines using the driver *elav-Gal4*, which is primarily used as a postmitotic neuronal driver with transient expression in neural precursor cells (Simcox Lab, unpublished; Berger *et al.* 2007; Hilgers *et al.* 2012). The *elav-Gal4* driver was not sufficient to produce neuronal cell lines and it was hypothesized that this was due to the expression of the driver too late in the development of the *Drosophila* nervous system (Simcox Lab, unpublished). As a result, out of the seven Gal4 drivers surveyed, I selected *scratch-Gal4* as my first neuronal driver because of the expression of *scrt* in most neuronal precursor cells; additionally, embryonic central nervous system expression patterns depicted that *scratch-Gal4* expression is present throughout the *Drosophila* embryo (Roark *et al.* 1995; Janelia Gal4 Stocks, Flylight Project, Bloomington Stock Center #47368).

In order to visualize the expression of the three selected Gal4 drivers, *scratch-Gal4*, *OK6-Gal4,* and *Toll-6-Gal4* in the *Drosophila* embryo and larvae, I used the reporter gene *UAS-GFP*. I set up crosses with the three drivers and *UAS-CD8-GFP* to visualize the respective Gal4 expression in the progeny larvae with green fluorescent protein (Figure 1). The Gal4 expression of the motor neuron-specific drivers was visualized in embryos by setting up crosses with *UAS-Ras^{V12}; UAS-GFP* (Figure 2).

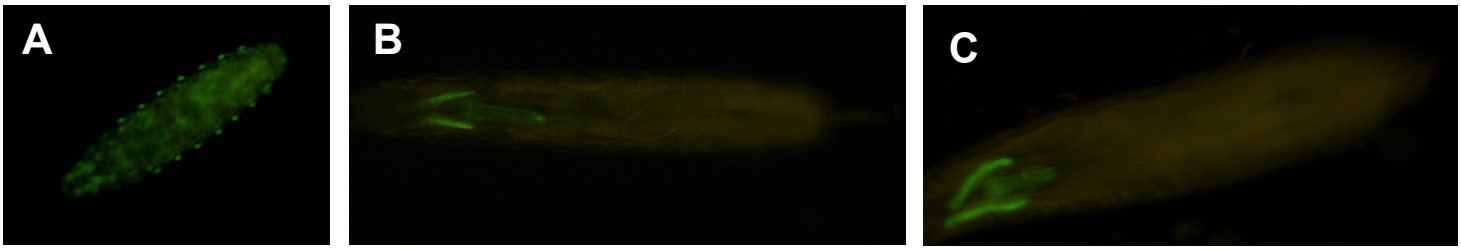

Figure 1. Gal4 driver expression visible in *Drosophila* larvae with green fluorescent protein. (A) *Scratch-Gal4* larvae show Gal4 expression throughout the central nervous system (B, C) *OK6-Gal4* and *Toll-6-Gal4* show Gal4 expression in the ventral nerve cord and salivary glands.

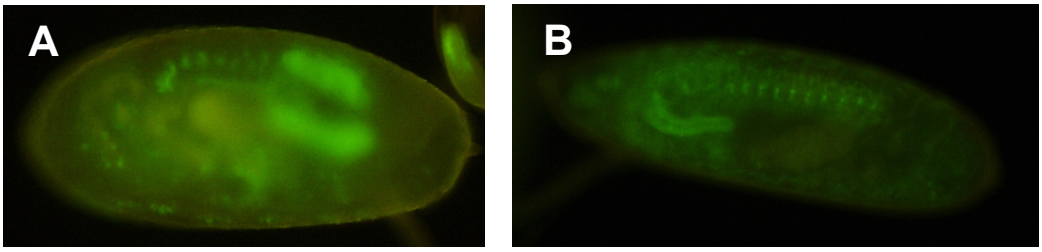

Figure 2. Motor neuron-specific Gal4 expression in *Drosophila* embryos. (A) *OK6-Gal4* embryo (B) *Toll-6-Gal4* embryo

Development of neural-specific primary cultures via targeted Ras^{V12} expression

After selecting *scratch-Gal4* as a pan-neural driver to test production of neural-specific continuous cell lines, I set up crosses using three UAS responder lines. All three responder lines have upstream activating sequences (UAS) upstream of a Ras oncogene (Ras^{V12}) in order to specifically promote the proliferation of cells of neuronal lineage. Each has additional transgenes, the function of which will be discussed in the relevant section. In progeny of the cross between *scratch-Gal4* and each UAS responder line, the Gal4 protein would bind to the upstream activating sequences, leading to the transcription and subsequent expression of Ras^{V12} in cells expressing *scrt*. We hypothesized that the growth and proliferation of the neuronal precursor cells expressing *scrt* would be promoted due to the expression of Ras^{V12}, providing these neural-specific cells with a survival advantage over other cell types that may exist in the primary culture (Simcox, unpublished).

The first UAS responder line used was *UAS-Ras^{V12}; UAS-GFP*. From the cross with *scratch-Gal4* and *UAS-Ras^{V12}; UAS-GFP*, the neural-specific cells expressing Ras^{V12} also express GFP, allowing for their visualization using fluorescent microscopy (Figure 3A). Eight primary cultures were produced from this cross. The next UAS responder line used was *UAS-Ras^{V12}, RMCE*, where RMCE stands for recombination mediated cassette exchange. RMCE is a system that allows for the transfection of single-copy transgenes into specific locations in the *Drosophila* genome (Manivannan *et al.* 2015). The creation of continuous cell lines of neuronal lineage that contain RMCE would provide the extra utility of allowing future researchers the resource to transfect these neural-specific cells with transgenes, such as those associated with a given disease phenotype, to easily study its pathways and expression in vitro. Ten primary cultures were produced from the cross of *UAS-Ras^{V12}, RMCE* with *scratch-Gal4*, one of which

reached confluence and was passaged. However, this first passage did not grow to confluence. Images depicting the growth of one primary culture from this cross are shown in Figure 3B, C, and D.

Since the crosses with *scratch-Gal4* utilizing Ras^{V12} alone to promote proliferation of the neural-specific cells did not prove to be successful, a third cross was attempted with *UAS-p35; UAS-*Ras^{V12}*, RMCE* flies. These flies, in addition to expressing the Ras^{V12} oncogene, and containing sites for RMCE, also express the baculovirus p35 protein. The p35 protein prevents the apoptotic death of p35-expressing cells; thus, the suppression of cell death via p35 was used to complement the proliferation of neuroblasts using oncogenic Ras^{V12} (Hay *et al.* 1994). 11 primaries were produced from this cross, of which three reached confluence. Similar to the passaged culture produced from the *UAS-*Ras^{V12}*, RMCE* cross, none of these three cultures survived beyond passage one. Images depicting the growth of one of these primaries, and its growth in passage one, are shown in Figure 3E, F, and G. The *scratch-Gal4* crosses and primaries produced are summarized in Table 2, along with the average days for the respective primaries to reach confluence.

Driver Line	Responder Line	Number of Primary Cultures	Average Days to Confluence
Scratch-Gal4 >	UAS-Ras^{V12}; UAS-GFP	8	N/A
	UAS-Ras^{V12}, RMCE	10	Day 78
	UAS-p35; UAS-Ras^{V12}, RMCE	11	Day 83

Table 2. Summary of *scratch-Gal4* crosses and average days to confluence of primary cultures.

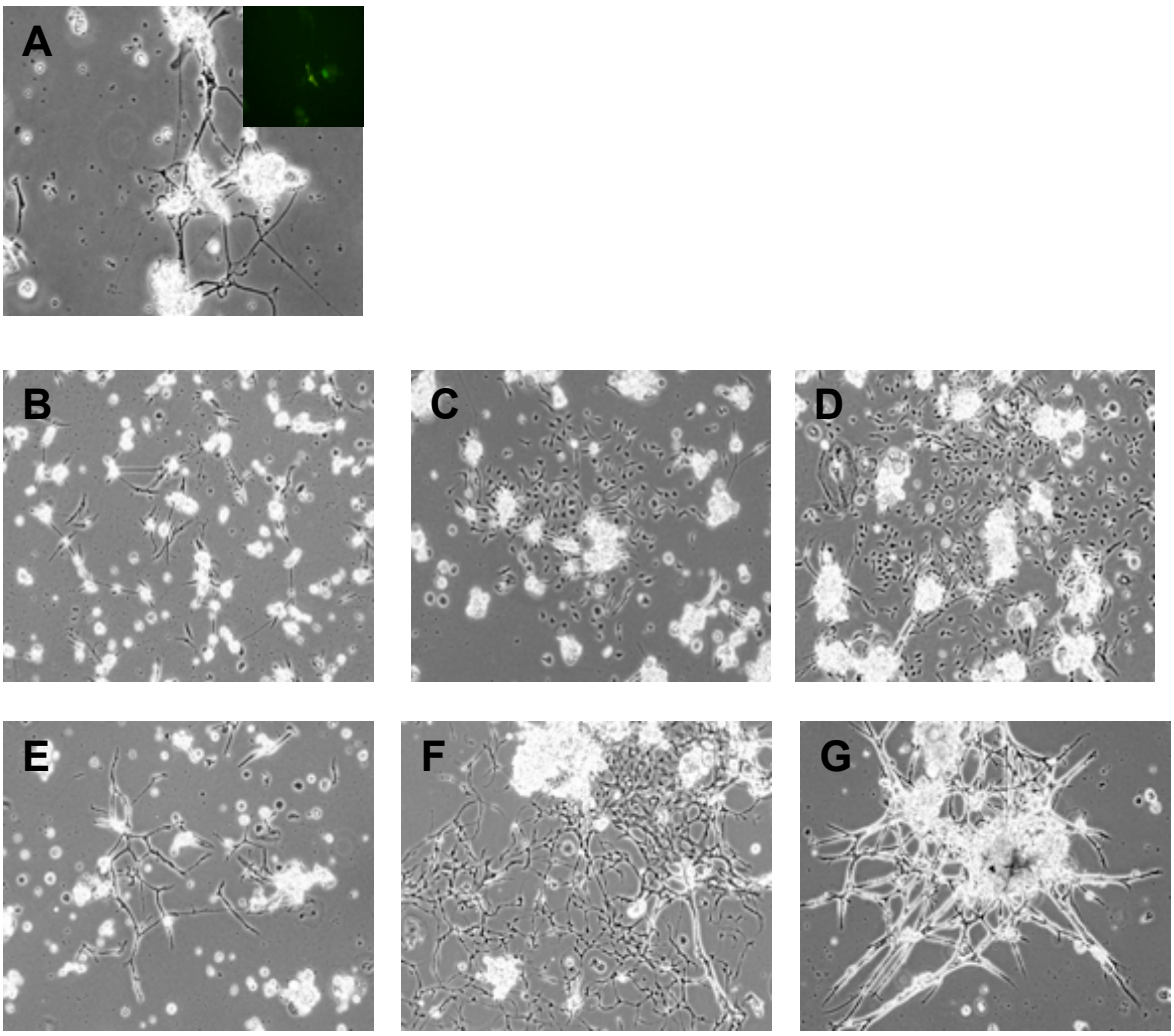

Figure 3. Primary cultures produced from *Scratch-Gal4* and three UAS responder lines expressing Ras^{V12}. (A) *Scratch-Gal4 > UAS-Ras^{V12}; UAS-GFP* primary at day 8 (B, C, D) *Scratch-Gal4 > UAS-Ras^{V12} RMCE* primary at days 4, 20, and 34 respectively (E, F) *Scratch-Gal4 > UAS-p35; UAS-Ras^{V12} RMCE* primary at days 20 and 71 respectively (G) *Scratch-Gal4 > UAS-p35; UAS-Ras^{V12} RMCE* primary passage 1 at day 13. All images at 10X magnification.

Development of motor neuron-specific primary cultures via targeted Ras^{V12} expression

The drivers *OK6-Gal4* and *Toll-6-Gal4* were selected to produce motor neuron-specific continuous cell lines, once again utilizing the oncogene Ras^{V12} to promote the proliferation of motor neurons in culture. These two driver lines were selected due to their expression in motor neurons. *OK6-Gal4* is expressed primarily in motor neurons and *Toll-6-Gal4* is expressed in motor neurons, the peripheral nervous system, and body wall sensory nervous system (Sanyal *et al.* 2003 and Sanyal 2009). Both driver lines were crossed with *UAS-p35; UAS-Ras^{V12}, RMCE.* Ten primary cultures were produced from the cross with *OK6-Gal4* and 11 primary cultures were produced from the cross with *Toll-6-Gal4*. None of the primary cultures reached confluence, but one primary culture from the cross with *OK6-Gal4* with several, large patches of cells was passaged into a 25 cm^3 flask. Images from representative primaries from each cross that depict the growth of cells with neuronal morphology into proliferative patches are shown in Figure 4.

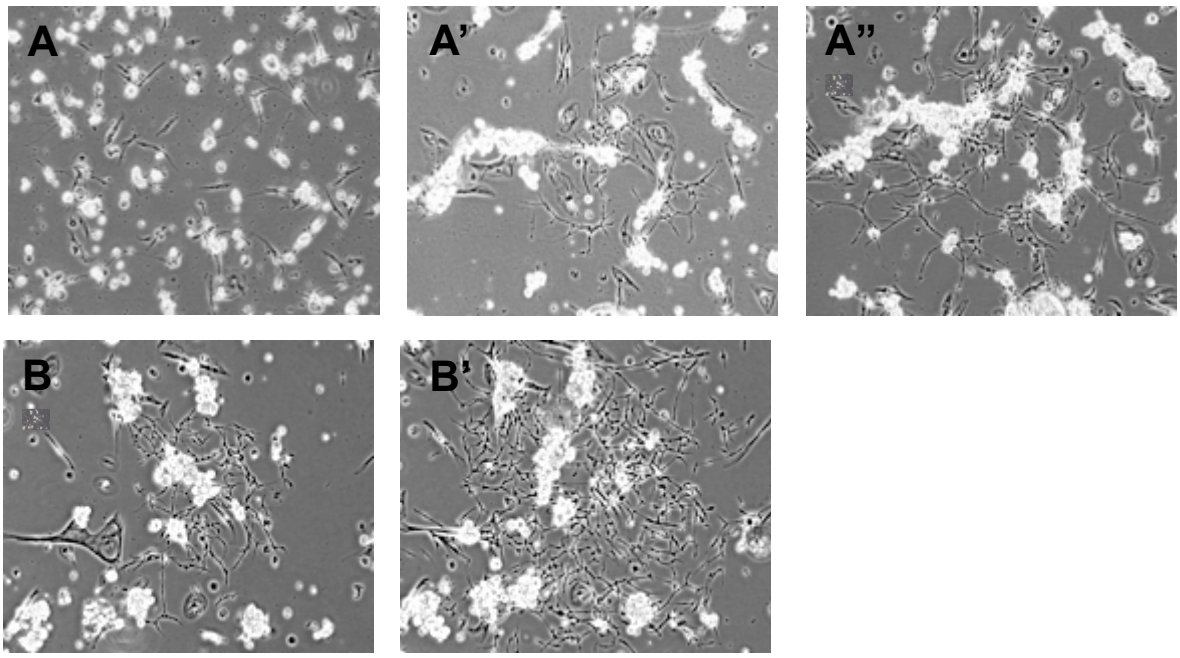

Figure 4. Primary cultures produced from crosses with the *OK6-Gal4* and *Toll-6-Gal4* motor neuron drivers and *UAS-p35; UAS-Ras^{V12}, RMCE*. (A, B, C) *OK6-Gal4 > UAS-p35; UAS-Ras^{V12}, RMCE* primary at days 4, 22, and 35 respectively. (D, E) *Toll-6-Gal4 > UAS-p35; UAS-Ras^{V12}, RMCE* primary at days 29 and 38 respectively. All images at 10X magnification.

Though the *OK6-Gal4* and *Toll-6-Gal4* drivers are largely motor neuron-specific, the produced primary cultures had cell patches containing cells with different neuronal-like morphology. For instance, there was variability in the diameter and length of extensions from the cell bodies in different patches of cells. Due to the diversity in morphology present in the primary cultures, it was of interest to find out which of the morphologies belonged to that of a motor neuron. To determine this, I crossed the *OK6-Gal4* and *Toll-6-Gal4* drivers with *UAS-Ras^{V12}; UAS-GFP*. As a result, in the produced primary cultures, the motor neurons with Ras^{V12} driving expression of OK6 and Toll-6 would be labeled with the GFP reporter and visible through fluorescent microscopy. Primary cultures were produced from this cross and GFP-expressing cells will be monitored in ongoing experiments.

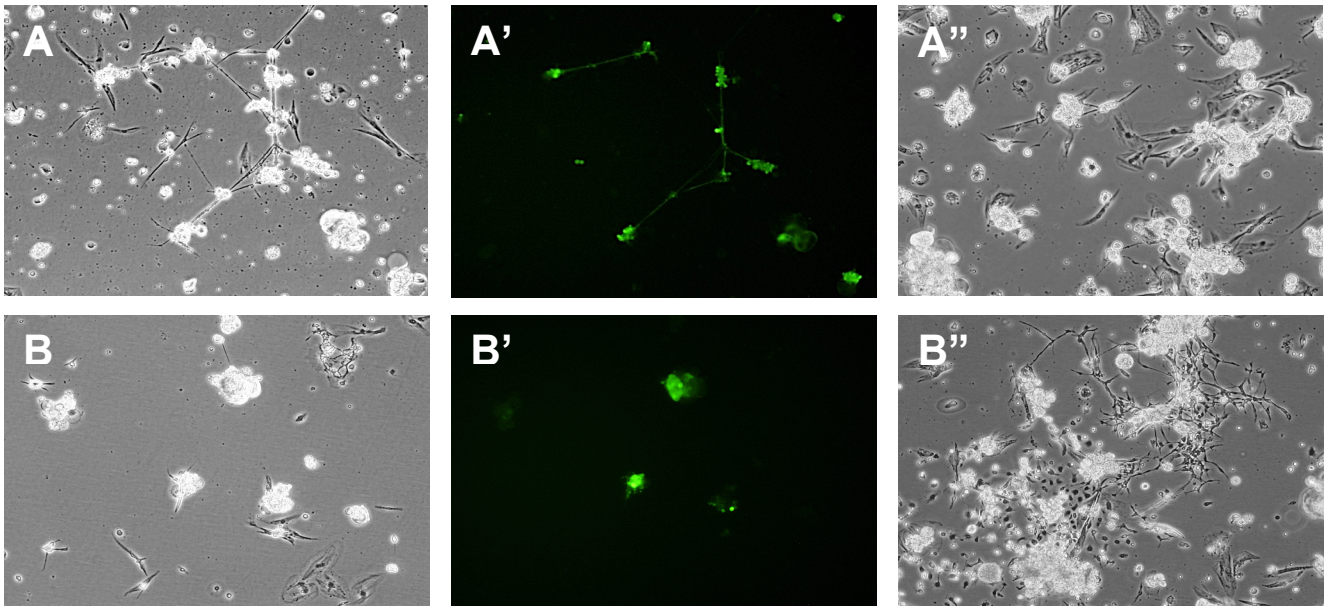

Figure 5. Primary culture produced from cross with the *Toll-6-Gal4* motor neuron driver and *UAS-Ras^{V12}; UAS-GFP*. (A, A') Phase image and GFP-expressing cells from a *Toll-6-Gal4 > UAS-Ras^{V12}; UAS-GFP* primary at day 19. (A") Phase image of *Toll-6-Gal4 > UAS-p35; UAS-Ras^{V12}, RMCE* confluent primary at day 57. (B, B') Phase image and GFP-expressing cells from a *OK6-Gal4 > UAS-Ras^{V12}; UAS-GFP* primary at day 19. (B") Phase image of *OK6-Gal4 > UAS-p35; UAS-Ras^{V12}, RMCE* confluent primary at day 63.

Development of neural-specific primary cultures via inhibition of Notch signaling

Inhibition of the Notch signaling pathway through RNA interference was utilized as a third strategy to produce neural-specific continuous cell lines. An experiment was set up using *UAS-Notch^{dsRNA}* flies, in which double-stranded RNA is transcribed that is complementary to the mRNA coded by the *Notch* gene in the fly, thus resulting in complementary basepairing, degradation, and silencing of the *Notch* gene (Boutla *et al.* 2001). These *UAS-Notch^{dsRNA}* flies were crossed with *Act5C-Gal4/TM6b, Tb* flies, which express cytoplasmic actin throughout many cell types in *Drosophila*. As a result, the progeny of the cross have inhibition of Notch signaling in the many cell types throughout the *Drosophila* body, theoretically allowing for the growth of the default cell type of Notch-controlled progenitor cells: neuronal cells. Primary cultures were produced from this cross and will be monitored for growth and morphology in

ongoing experiments.

RR1 and RR2 cells express neuronal-specific markers

Two cell lines, named Ras-attP-L1 (RR1) and Ras-attP-L2 (RR2) respectively, were established by the Simcox laboratory from a cross between *Act5C-Gal4/TM6* flies and *UAS-Ras^{V12}; P[attP.w+.attP]JB89B/TM3* flies (Manivannan *et al.* 2015). Since *Act5C-Gal4* is a cytoplasmic actin gene that is expressed throughout many cell types in *Drosophila melanogaster*, it was expected that these established cell lines RR1 and RR2 would exhibit multiple lineage-specific cell types. However, both the RR1 and RR2 cells appeared to exhibit neuronal-specific morphology, with neurite outgrowths and extensions. To characterize the lineage of these cell lines, I conducted immunohistochemistry staining of the cells using three neuronal-specific markers: 22C10, Fasciclin-II, and horseradish peroxidase (HRP) (Figures 5, 6, and 7). These three markers are all expressed in different subsets of the *Drosophila* nervous system. The 22C10 antigen is expressed by central nervous system neurons and all neurons in the peripheral nervous system; specifically, it is found in all cellular compartments, including the soma, axon, and the dendrite (Hummel *et al.* 2000). Fasciclin-II is the fly ortholog of neural cell adhesion molecule and is expressed in a subset of fasciculating axons in the nervous system (Squire 2008). The HRP antigen is expressed in sensory neurons, peripheral nerves, and central nervous system fiber tracks (Jan and Jan 1982). An epithelial cell line derived from a *warts* (wts) tumor suppressor mutant was used as a negative control (Simcox 2013). Wts was identified to be an epithelial cell line based on positive immunostaining for E-cadherin, a commonly used marker of epithelial cells (Simcox 2013).

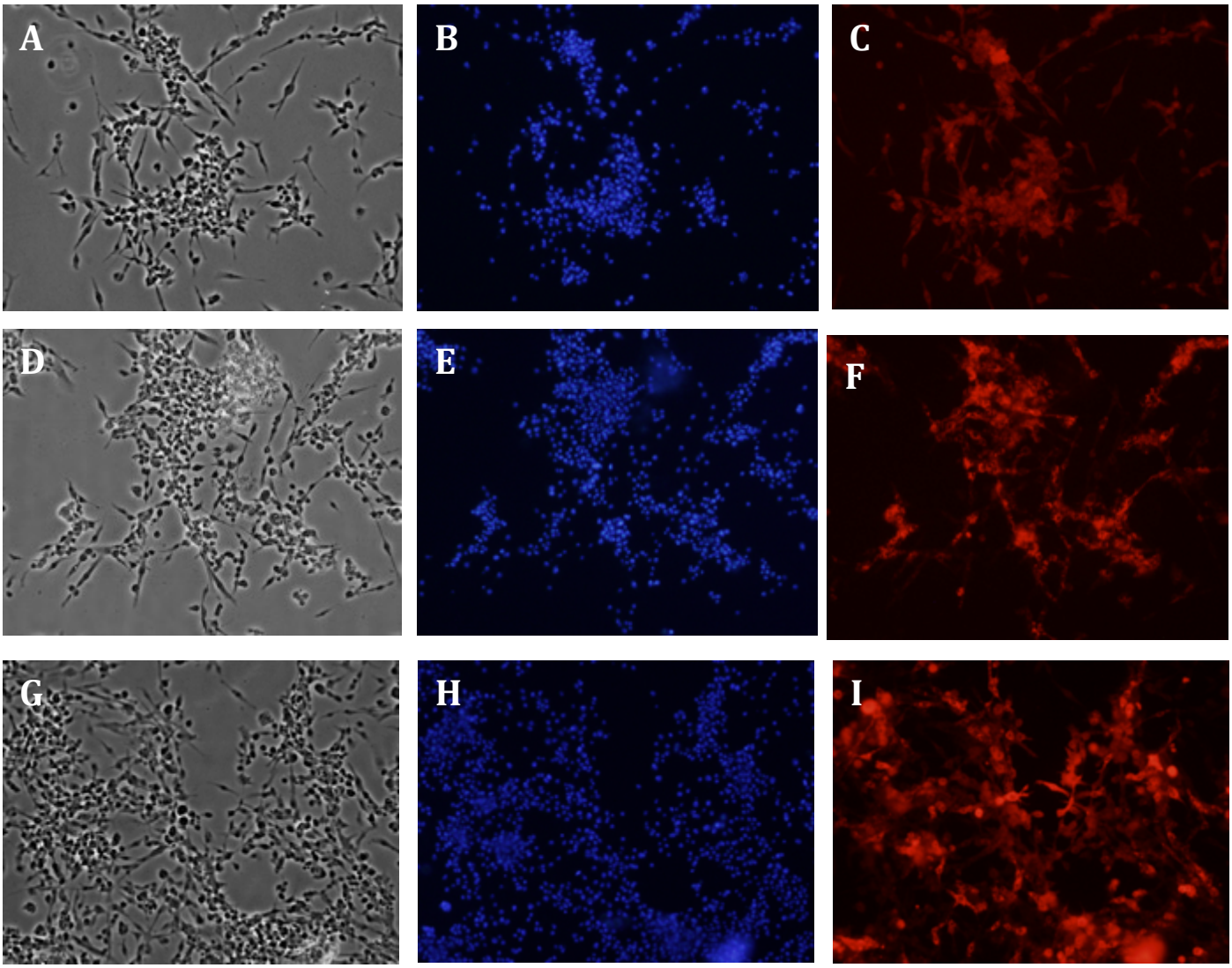

Figure 6. Ras-attP-L1 (RR1) cells express several neuronal-specific markers. (A, B, C) Cells under phase-contrast, DAPI fluorescence, and 22C10 staining respectively (D, E, F) Cells under phase-contrast, DAPI fluorescence, and Fasciclin-II staining respectively (G, H, I) Cells under phase-contrast, DAPI fluorescence, and HRP staining respectively. All images at 20X magnification.

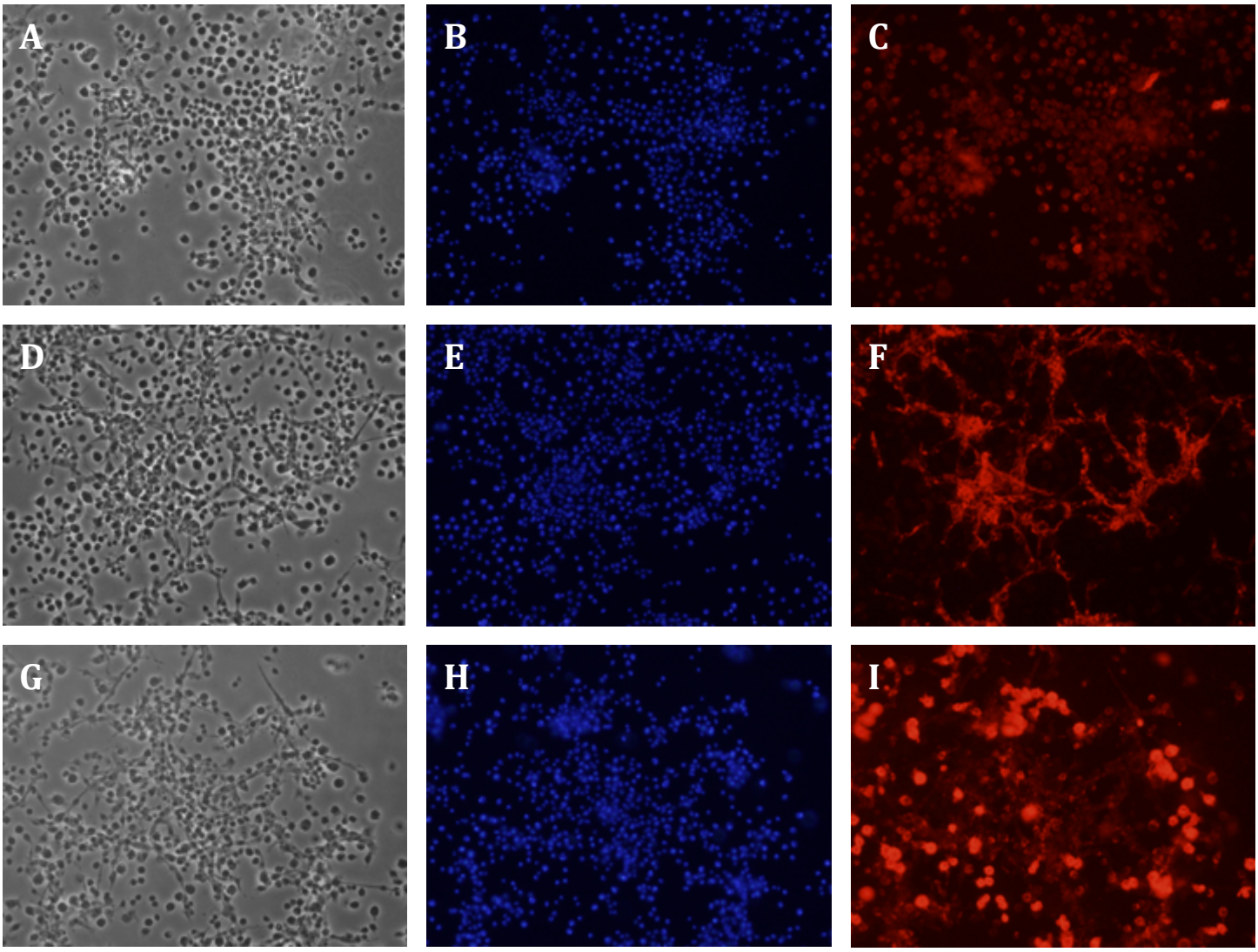

Figure 7. Ras-attP-L2 (RR2) cells express several neuronal-specific markers. (A, B, C) Cells under phase-contrast, DAPI fluorescence, and 22C10 staining respectively (D, E, F) Cells under phase-contrast, DAPI fluorescence, and Fasciclin-II staining respectively (G, H, I) Cells under phase-contrast, DAPI fluorescence, and HRP staining respectively. All images at 20X magnification.

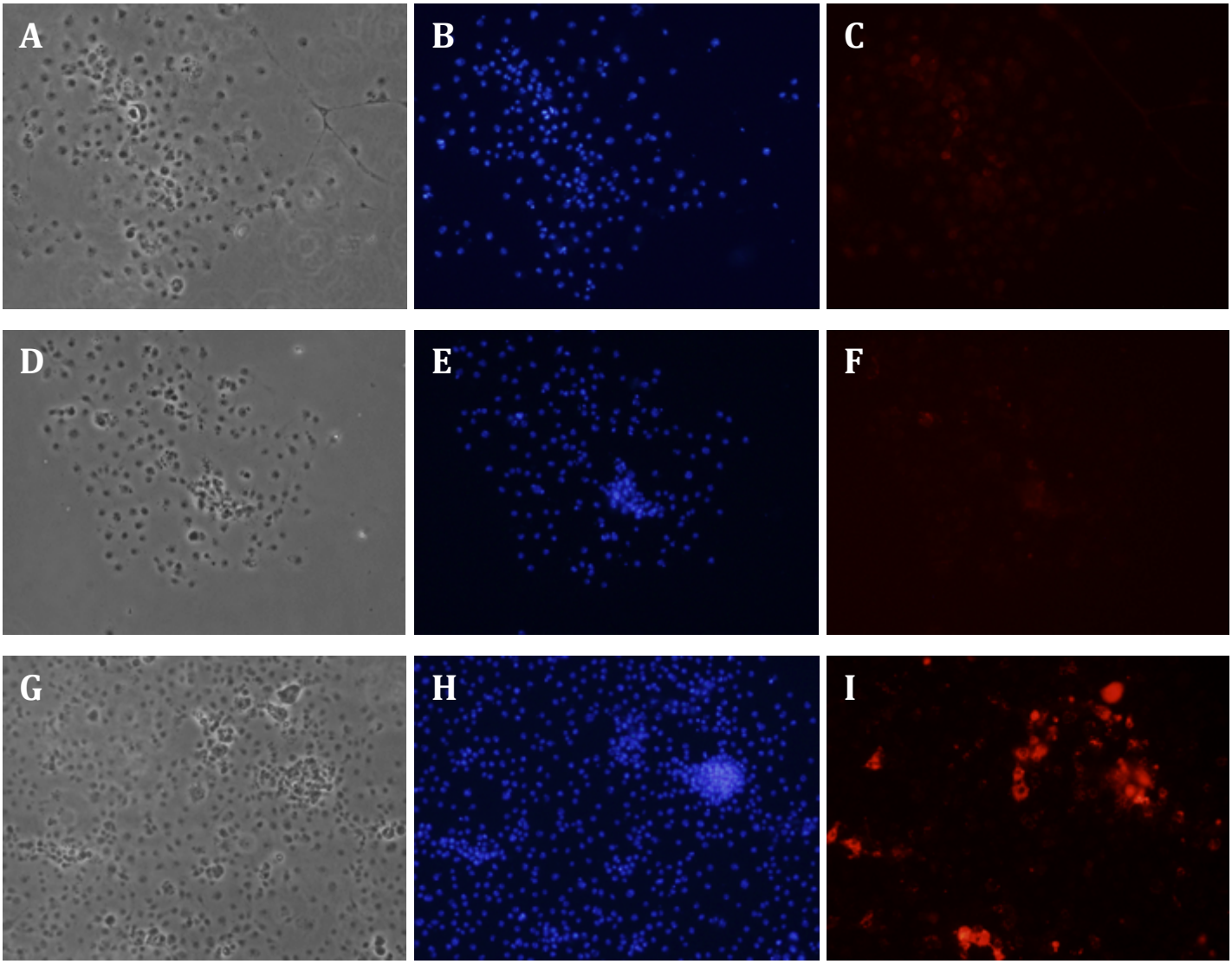

Figure 8. Wts cells do not express neuronal-specific markers. (A, B, C) Cells under phase-contrast, DAPI fluorescence, and 22C10 staining respectively (D, E, F) Cells under phase-contrast, DAPI fluorescence, and Fasciclin-II staining respectively (G, H, I) Cells under phase-contrast, DAPI fluorescence, and HRP staining respectively. All images at 20X magnification.

Discussion

Pan-neuronal expression of Ras^{V12} with scratch-Gal4 did not result in continuous cell line generation

Three different neural- and motor neuron-specific Gal4 drivers were used in combination with UAS responder lines expressing oncogenic Ras^{V12} and the baculovirus p35 protein to produce primary cultures; however, none of the primary cultures developed into cell lines. Of the four primary cultures produced from crosses with *scratch-Gal4*, none survived beyond passage one. One explanation of these results could be the relative strength of Gal4 expression. Larval images showing the GFP expression of each of the Gal4 drivers displays the relatively weak expression of *scratch-Gal4* throughout the central nervous system in *Drosophila melanogaster* (Figure 2). It is possible that *scratch-Gal4* expression is too weak to drive proliferation of neuroblasts in culture, despite the fact that *scratch-Gal4* is believed to be involved with early neurogenesis in the fly.

Motor neuron-specific expression of Ras^{V12} with OK6-Gal4 and Toll-6-Gal4 did not result in continuous cell line generation

In contrast to *scratch-Gal4* expression, the expression of *OK6-Gal4* and *Toll-6-Gal4* appears to be stronger and specific to the ventral nerve cord and larval salivary glands (Figure 2). Despite these strong and specific expression patterns, the *OK6-Gal4* and *Toll-6-Gal4* crosses with *UAS-p35; UAS-Ras^{V12}, RMCE* were not successful in producing continuous cell lines.

Promoting cell survival with baculovirus p35 did not enhance cell line production

In the production of primary cultures, UAS responder lines containing Ras^{V12} alone, as well as those containing Ras^{V12} together with the baculovirus protein p35, were used in crosses with *scratch-Gal4*. When directly comparing the *scratch-Gal4 > UAS-*Ras^{V12}*, RMCE* primary cultures with *scratch-Gal4 > UAS-p35; UAS-*Ras^{V12}*, RMCE* primary cultures, there was one primary from the first cross that reached confluence, and three primaries from the latter cross that reached confluence. However, given the fact that none of these primary cultures survived past passage one, and the small sample size of primary cultures that reached confluence, it is difficult to assess whether or not the inclusion of p35 was truly beneficial. Moreover, these four primaries reached confluence after about 80 days; research from other members in the Simcox Lab who are working on developing similar lineage-specific continuous cell lines using Ras^{V12} has indicated that successful cultures typically reach confluence after only about 30 to 40 days (Simcox, unpublished).

RR1 and RR2 cells express markers of neuronal lineage

The two lines, RR1 and RR2, which were produced from a cross with *Act5C-Gal4/TM6b* and *UAS-*Ras^{V12}*; P[attP.w+.attP]JB89B/TM3*, stained positively for three neuronal-specific markers: 22C10, Fasciclin-II, and HRP. The negative control, the epithelial cell line wts, did not stain positively for the same three markers. The RR1 and RR2 cell lines, therefore, have characteristics of the neuronal lineage, identifying them as promising candidates for future research studying mechanisms of the nervous system. These cells also contain sites for RMCE, allowing for increased flexibility for studying disease phenotypes and mechanisms as a transgene can be inserted into the cell lines through ϕC31 recombination (Manivannan *et al.* 2015). Though

the RR1 and RR2 cell lines may have great utility for future research, there are still limitations in the replicability of creating such neural-specific cell lines, as RR1 and RR2 were produced from ubiquitous Ras^{V12} expression. In regard to refining a method to produce cells of neuronal lineage, future studies could be aimed at screening for pan-neural or motor neuron-specific Gal4 drivers with stronger expression, as well as trying alternative protocols, such as the direct dissection and plating of multiple larval *Drosophila* brains into primary culture or the use of other genetic manipulations. Moreover, ongoing and future studies are aimed at examining the potential of inhibiting Notch signaling to produce neural-specific continuous cell lines.

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